

The Actin-associated Protein Palladin Is Required for Development of Normal Contractile Properties of Smooth Muscle Cells Derived from Embryoid Bodies*^[5]

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Palladin is a widely expressed actin-associated protein localized at stress fibers, focal adhesions, and other actin-based structures, playing a significant role in cell adhesion and cell motility. Knockout of *Palladin* in mice is embryonic lethal, demonstrating the importance of Palladin in development yet its role in the vasculature is not known. In the present study, smooth muscle cell (SMC) markers, such as myosin, actin, caldesmon, calponin, and LPP, were down-regulated in embryoid bodies (EBs) derived from embryonic stem cells lacking *Palladin*. Transgenic embryonic stem cell lines were generated that stably expressed a puromycin-resistance gene under the control of a SM α -actin (*SMA*) promoter. Negative selection was then used to purify SMCs from EBs. Purified SMCs expressing multiple SMC markers were designated APSCs (*SMA*-puromycin-selected cells). *Palladin* null APSCs express significantly less myosin, actin, calponin, and h-caldesmon. The filamentous (F) to globular (G) actin ratio, known to regulate myocardin family transcription factors, was also decreased. *Palladin* null APSCs showed increased cell adhesion and decreased cell motility. Importantly, *Palladin* null APSCs within collagen gels generated less maximum contractile force when stimulated with endothelin-1, sphingosine 1-phosphate (S1P), and thrombin. Myosin light chains (MLC20) were phosphorylated by lysophosphatidic acid to the same extent in *Palladin* null and wild type APSCs but myosin content/total protein was reduced by >50%, consistent with the observed decreases in contractility. All together, these results suggest that *Palladin* is essential for expression of the full complement of contractile proteins necessary for optimal force development of SMCs derived from EBs.

The contractile state of smooth muscle regulates the normal function of most hollow organs, the airway and the vasculature. Disregulation can result in diseases such as hypertension and asthma. Contractility of smooth muscle depends on myosin regulatory light chain phosphorylation and cross-

bridge cycling, as well as expression and organization of the contractile apparatus. Palladin is a key regulator of actin organization. It localizes to focal adhesions, cell-cell junctions, dorsal and circular ruffles, growth cones, and other actin-based cellular structures, and is required for the maintenance of normal stress fibers in cultured cells (1–3). Other palladin family members expressed in skeletal muscle are important for sarcomere integrity and mutated forms are associated with inherited muscular disorders. We have found that palladin is highly expressed in smooth muscle (SM) (4), raising the possibility that palladin could be important, as in striated muscles, for the organization of the SM cytoskeleton and thus contraction. Loss of *Palladin* retards motility of SMCs (4), primary neurons and neuroblastoma cells (5), invasive carcinoma cells (6), and fibroblasts (7). Palladin is rapidly up-regulated when fibroblasts differentiate into contractile myofibroblasts (8). Palladin serves as a scaffold for multiple actin-binding proteins, signaling molecules, and also is an actin cross-linking protein (9). It localizes to dense bodies in differentiated SM tissues and has a migratory role in response to environmental cues such as following vascular injury (4). SMC motility involves reorganization of the cytoskeleton, relies on the recruitment of multiple signaling and adaptor proteins to focal or fibrillar adhesion sites that reiteratively form, deconstruct, and regenerate themselves, allowing cells to detach and reattach to the existing and provisional matrix on which they exert a tractional force (10). Thus, it appears that palladin plays an important role in organizing actin arrays within migrating cells, through both direct and indirect molecular mechanisms.

We have previously shown that palladin interacts with the focal adhesion protein lipoma preferred partner (LPP) at focal adhesions in cultured cells and dense bodies in SM tissues (4). They both play a significant role in SMC migration and spreading and that expression of both LPP and Palladin, like SMA, is increased by angiotensin II, regulated by actin dynamics, modulated by focal adhesion kinase, and appear in the neointima of injured vessels. We have also reported, in a model system of

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^[5] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S3.

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² The abbreviations used are: SM, smooth muscle; EB, embryonic bodies; SMC, smooth muscle cell; ESC, embryonic stem cells; SMA, smooth muscle α -actin; APSC, SMA-puromycin-selected cells; MLC, myosin light chain; GPCR, G protein-coupled receptor; LPP, lipoma preferred partner; SM-MHC, smooth muscle-myosin heavy chain; NMIIA, non-muscle myosin IIA; NMIIIB, non-muscle myosin IIB; MRTF, myocardin-related transcription factors; SRF, serum response factor; RT, reverse transcriptase; PBS, phosphate-buffered saline; KO, knock-out; WT, wild type; S1P, sphingosine 1-phosphate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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SMC differentiation, that Palladin and LPP, like other SM contractile proteins, are up-regulated upon differentiation and modulated by myocardin SRF, coactivator of transcription in SM and cardiac muscle, and by the Rho/ROCK signaling pathways (4). The precise role of *Palladin* in embryonic development and vascular SM is elusive. Inactivation of *Palladin* leads to lethality at embryonic day 15.5 due to severe defects of cranial neural tube closure and germination of liver and intestine (11, 12) thus indicating that *Palladin* plays a critical role in embryonic development. However, because of the early death of *Palladin* knock-out mouse embryos, its role in SMC differentiation and development of contractile properties has not been determined. Interestingly, Palladin is up-regulated in neural crest cells, which is one of multiple embryonic lineages giving rise to SMCs (13). The present studies were focused on determining the effects of *Palladin* knock-out on development of SMC from embryonic stem cells using a novel *in vitro* embryoid model of differentiation previously reported by our laboratories (14). Of interest, we show for the first time that *Palladin*-deficient ESCs can form EBs containing SMCs but these cells showed marked reductions in expression of multiple SMC marker genes including contractile proteins. In addition, *Palladin* knock-out ESC/EB-derived SMCs exhibited reduced contractile force development in response to agonist stimulation reflecting a reduction in myosin filament content and/or a disorganized actin network.

EXPERIMENTAL PROCEDURES

ESC Culture—ESCs were maintained in Dulbecco's modified Eagle's medium containing 15% fetal bovine serum, 0.1 mM β -mercaptoethanol, 0.1 mM minimal essential medium nonessential amino acids, 0.2 mM L-glutamine, and 100 units/ml leukemia inhibitory factor. ESCs were initially cultured on a feeder layer of irradiated embryonic fibroblasts (14).

Selection of Transgenic ESCs and EB Formation—The heterozygous *Palladin* null ESCs (BayGenomics, San Francisco, CA) derived by gene trap insertion in intron 20 of *Palladin* were selected with neomycin and genotyped with RT-PCR to identify the homologous and heterozygous *Palladin* null ESCs. ESC/EBs were formed by aggregating the ESCs in hanging drops for 72 h and then cultured in suspension for 3 days. On day 6, the ESC/EBs were plated onto 0.1% gelatin-coated surfaces and allowed to attach for 24 h, then were supplemented with 10 nM all-*trans* retinoic acid (14). The EBs were harvested on days 7, 10, 15, and 28 for real time RT-PCR and Western blotting.

ESC-derived SMC Isolation—ESCs were electroporated with a plasmid containing a puromycin-*N*-acetyltransferase under the control of a SMA promoter (14, 15). Stable puromycin-*N*-acetyltransferase ESCs colonies were selected by puromycin and the presence of puromycin-*N*-acetyltransferase was confirmed by PCR. EBs were generated as described above. SMA expressing SMCs were isolated by selection with puromycin. These SMC-like cell lines were designated as SMA-puromycin selected cells (APSCs).

Smooth Muscle Collagen Fiber Preparation and Isometric Force Measurement—Contractile function was evaluated by incorporating cultured cells into collagen gels as previously described (15, 16). In brief, cultured cells were trypsinized and resuspended at a density of 2×10^6 cells/ml in neutralized collagen I solution containing 1.2 mg/ml of collagen in PBS. The collagen solution was transferred into a rectangular trough ($17 \times 5.5 \times 1.7$ mm) with a 1.6-mm diameter Teflon pole near each end. Culture medium was added to the dishes and specimens cultured for 14 days. The medium was changed every other day.

The rod-shaped fibers was cut into 4-mm long strips, and then cut in half longitudinally. For isometric force measurements one end of the segment was tied to a force transducer and the other to a micromanipulator using monofilament silk thread as previously described (14, 17). The fibers were stretched to 1.2 times slack length and equilibrated in the normal Krebs-bicarbonate solution for at least 1 h before experiments. Strips were oxygenated and kept at 30 °C in Krebs-bicarbonate buffer throughout the experiments. The contractile responses to vasoactive agonists including high potassium, sphingosine 1-phosphate (S1P), thrombin, endothelin-1, and the myosin phosphatase inhibitor calyculin A (Calbiochem, San Diego) were as indicated in the figures.

Real Time RT-PCR—Total RNA was extracted with TRIzol and cDNA was generated with a cDNA synthesis kit as previously described (4, 18). Quantitative RT-PCR was performed as previously published (14, 18, 19). Primer and probe sequences for *Palladin* (both 140 and 90 isoforms) were as follows: sense, 5'-AGCATGCACCAGGATAATCA-3'; antisense, 5'-CAG-GACACAATGCCTGCTT; probe, 5'-AAGACGCTGGGT-GGTACACT.

Western Blot—ESCs or EBs were lysed in RIPA buffer supplemented with 1% of proteinase inhibitor mixture. The extracts were cleared at $15,000 \times g$ for 10 min at 4 °C. The equal amounts of protein were subjected to SDS-PAGE. Western blotting was performed as published (4, 18). The sources of antibody used: LPP polyclonal antibody, ImmunoGlobe, Germany; palladin polyclonal antibody, a generous gift from Dr. Carol Otey, University of North Carolina. This antibody recognizes the 140- and 90-kDa palladin isoforms based on Western blotting. MLC20 polyclonal antibody (a generous gift from Dr. Kris Kamm from the University of Texas Southwestern Medical Center), non-muscle myosin IIA polyclonal antibody (Sigma), non-muscle myosin IIB monoclonal antibody (Hybridoma bank, University of Iowa), SMA, h- and l-caldesmon, and vinculin monoclonal antibodies (Sigma). Calponin antibody as previously described (20). SM myosin heavy chain SM1 and SM2 polyclonal antibodies are a generous gift from Drs. Ann Martin, University of Cincinnati, and Lori Walker, University of Colorado. All other antibodies are from Santa Cruz Biotechnology (Santa Cruz, CA).

Globular (G):Filament (F)-Actin Ratio Analysis—The concentration of F-actin and G-actin in APSCs was measured as previously described (4).

Measurement of Myosin Light Chain (MLC) Phosphorylation—The subconfluent APSCs were serum starved overnight, stimulated with 1 μ M lysophosphatidic acid for 0, 1, 2.5, and 5 min,

and fixed with 10% trichloroacetic acid. The fixed samples were incubated on ice for 1 h, and collected by centrifugation at $7000 \times g$ for 10 min, then thoroughly washed with acetone. The dried samples were solubilized in urea gel buffer at room temperature. Equal amounts of protein were subjected to SDS-PAGE, and blotted with MLC20 polyclonal antibody (21).

Indirect Immunofluorescence—Cultured cells were fixed with 4% paraformaldehyde and permeabilized with 0.03% Triton X-100 in PBS, rinsed and blocked for 1 h in PBS containing 3% bovine serum albumin and 1 mM sodium azide, and then incubated with an rabbit anti-LPP polyclonal antibody (1:800), anti-SMA monoclonal antibody (1:500), and anti-Palladin polyclonal antibody (1:800) diluted in blocking solution for 2 h at room temperature or overnight at 4 °C. Secondary antibodies were Alexa 594-conjugated goat anti-rabbit IgG (1:1000; Molecular Probes) and Alexa 488 goat anti-mouse IgG (1:1000) in blocking buffer (Jackson ImmunoResearch, West Grove, PA). Some cells were labeled for 1 h with Alexa 488-conjugated phalloidin (1:1,000), and Alexa 594-conjugated DNase I (1:1000, Molecular Probes) for G-actin. Cells were washed four times for 5 min with PBS before being mounted with Aqua Poly/Mount (Polysciences, PA). Confocal images were obtained on an Olympus FV300 microscope.

Electron Microscopy—Collagen gels integrated with wild type and *Palladin* knock-out APSCs were fixed with 2% glutaraldehyde in 0.075 M cacodylate buffer with 4.5% sucrose, pH 7.4, overnight at 4 °C, washed three times with 0.075 M sodium cacodylate buffer (22), post-fixed in 2% osmium in 0.05 M cacodylate buffer followed by 1% tannic acid, and subsequently saturated uranyl acetate. Finally, the collagen gels were dehydrated in graded alcohols and embedded in Spurr's resin. Thin sections were examined in a Phillips CM12 electron microscope and images captured with an SIA 8C digital camera (Deluth, GA) CCD camera.

RESULTS

Palladin Was Required for the Induction of SMC Marker Genes within ESC-embryoid Bodies—Two independent clones of homozygous *Palladin* knock-out ES cells were isolated from heterozygous *Palladin* knock-out ESCs by use of high dose G418 screening. The genotype of clones was assessed by RT-PCR for the knock-out *Palladin* allele, and by Western blotting for Palladin protein expression (Fig. 1, A and B). Wild type, heterozygous knock-out, and homozygous *Palladin* knock-out ES cell lines all showed expression of the ESC markers, Oct4, Sox2 and SSEA-1 (stage specific embryonic antigen-1, Fig. 1C) confirming maintenance of pluripotency. The *Palladin* knock-out cells were then tested for their ability to differentiate into SMCs in the context of EBs. The homozygous and heterozygous ESCs were capable of forming EBs similar in size to wild type ES cells (data not shown).

In wild type EBs induction of SMC marker genes, including *SMA*, *SM22*, and *SM-myosin heavy chain (SM-MHC)*, has been previously reported (14, 23). *Palladin* mRNA was induced in EBs at day 20 (Fig. 1D). Of major interest, induction of these SMC marker genes as well as the *Lpp* gene was dramatically decreased in *Palladin* knock-out ESCs derived EBs at day 28 (Fig. 2A). There was no detectable change in the expression of

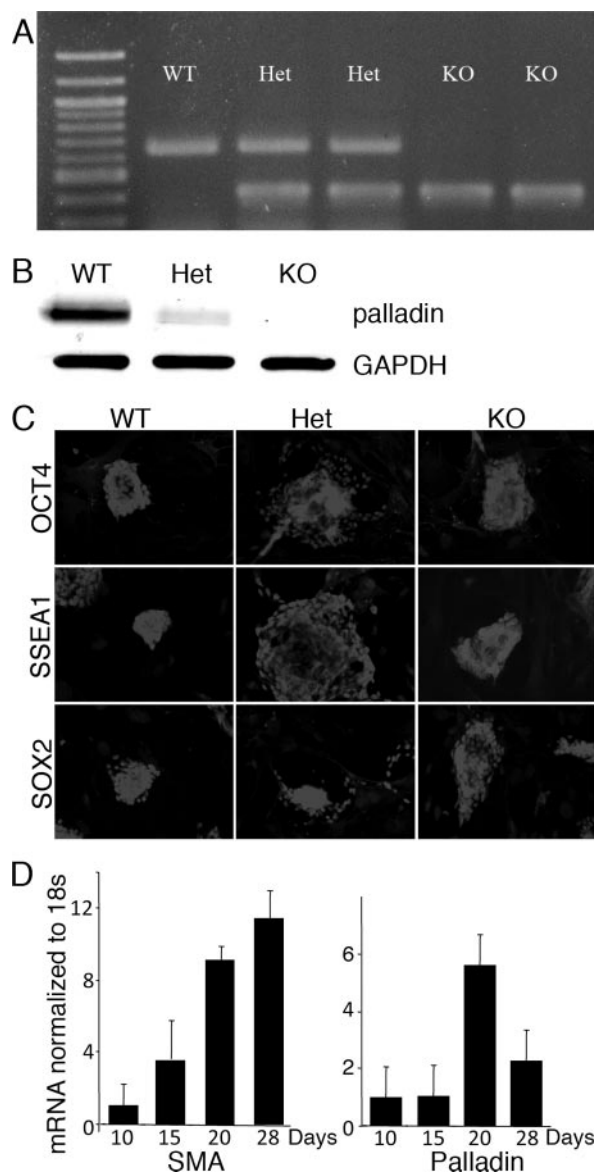


FIGURE 1. Characterization of *Palladin* knock-out ES cells. A, genotyping of *Palladin* knock-out ESC by RT-PCR. B, Western blotting confirms *Palladin* depletion in ESCs. C, *Palladin* knock-out ESCs were stained with ESC markers, Oct4, Sox2, and SSEA-1 to document retention of stem cell pluripotency. D, *Palladin* is induced during SMC differentiation in wild type mouse ESCs derived from EBs, and the expression of *Palladin* and *SMA* was determined by real time RT-PCR at days 10, 15, 20, and 28.

SMC genes at days 7 and 10 (data not shown). In heterozygous *Palladin* knock-out ESC-derived EBs, the SMC genes were also partially decreased.

SMC Derived from *Palladin* Knock-out ESC Showed Diminished Expression of SM Contractile Proteins—ESCs are pluripotent because they develop into a variety of cell types *in vivo* when injected into a developing blastocyst (24). *In vitro*, when placed in aggregates, ESCs form EBs that can recapitulate many developmental processes (24, 25). As part of their developmental program, EBs have been shown to form regions of spontaneously contracting SMCs (14, 26) (data not shown). Although these beating regions vary between preparations, they were observed in EBs derived from both *Palladin* null, heterozygous *Palladin* knock-out, and wild type ESCs. In *Palladin* homozy-

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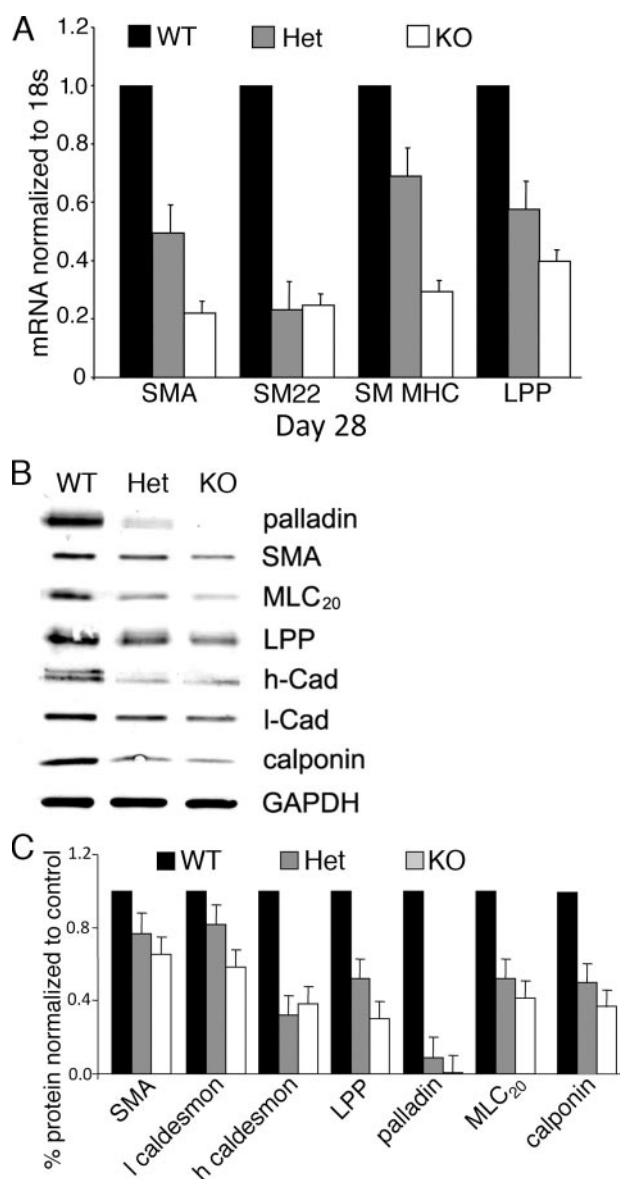


FIGURE 2. *A*, the expression of SMC differentiation marker genes is attenuated in embryoid bodies derived from *Palladin* knock-out homozygous ESCs. The wild type (*WT*), heterozygous (*Het*), and homozygous ESCs (*KO*) were induced to differentiate into embryoid bodies, and the expression of *SMA*, *SM22*, and *SM-MHC* at day 28 was determined by real time RT-PCR. *B*, the expression of contractile proteins was reduced in embryoid bodies at day 28. Western blotting shows the expression of SM contractile proteins in the wild type (*WT*), heterozygous (*Het*), and homozygous (*KO*) EBs. Equal amounts of protein were loaded for each. *C*, results of quantitative analysis of SM contractile proteins. The expression is normalized to GAPDH and the values for *Het* and *KO* cells normalized to that of controls.

gous knock-out EBs at day 28, the expression of SMC contractile proteins, MLC₂₀, caldesmon, and calponin was significantly decreased at both the mRNA and protein levels compared with wild type (Fig. 2). The expression of these contractile proteins was also partially decreased in the *Palladin* heterozygote EBs (Fig. 2). These results and those below on the APSCs suggest that palladin was essential for full SMC contractile protein induction during embryo development.

SMC Derived from Palladin Knock-out ESC Exhibited Reduced Force Generation When Aggregated within Collagen

Gels—To test the role of *Palladin* in SMC contractile regulation, we purified SMCs from ESC-derived EBs. The SMA-puromycin-*N*-acetyltransferase constructs were transfected into wild type and *Palladin* homozygous knock-out ESCs. The ESC clones that stably integrated the puromycin resistance gene under the control of the promoter-enhancer region of the *SMA* were amplified. EBs were generated from the transgenic ESC lines. At day 28 the EBs were disaggregated using enzyme digestion into a single cell suspension and plated in the presence of puromycin continually for 3 days. It has been shown that the *SMA* promoter-enhancer region is sufficient to completely recapitulate expression patterns of endogenous genes in transgenic mice and in EBs (14). The surviving puromycin-selected SMA expressing cells were designated as APSCs. Around 10% of plated cells survived overnight selection. The purity of APSCs was confirmed with SMA staining (data not shown). As shown in Fig. 3, *A* and *B*, the protein expression of SMA and the SM specific protein, LPP, was reduced in *Palladin* knock-out versus wild type APSC. Based on the absence of mRNA and Palladin protein detected by Western blotting, the residual Palladin fluorescence signal in the *Palladin* KO cells likely reflects nonspecific staining with this antibody. Protein levels of MLC₂₀, h-caldesmon, and calponin were reduced in the *Palladin* KO versus wild type APSCs (Fig. 3, *B* and *C*). SM-MHC was identified on Western blots with antibodies specific to SM1 and SM2, which do not react with non-muscle myosin II. Both SM1 and SM2 expression was decreased in *Palladin* null APSCs, whereas non-muscle IIA and IIB were unchanged (Fig. 3*D*). We incorporated the same number of wild type and *Palladin* knock-out APSCs into collagen gels (Fig. 4*A*). The central region of the collagen muscle fibers were cut into 4-mm long sections and split in half longitudinally for contractility studies. Thus each strip had the same dimensions, the same number of cells based on phalloidin staining, and the same number of cells counted following enzymatic dissociation of the reconstituted muscle fibers, and finally, Western blotting showed the same contents of GAPDH in WT and KO APSCs in the collagen gels (supplemental Fig. S1). Strips were mounted on a force transducer (Fig. 4*A*), and the contractile response to agonists was studied. Reconstituted muscle fibers generated from wild type APSCs had potassium-induced contractions (120 mM KCl) similar to intact SM tissue (14, 16). The muscle fibers contracted in response to S1P (1 μ M), thrombin (1 μ M), and endothelin-1 (10 nM). Finally, addition of the myosin phosphatase inhibitor, calyculin A (100 nM), was added to generate maximal MLC₂₀ phosphorylation and force. Maximum calyculin-induced force generated by *Palladin* knock-out APSCs was significantly less ($38 \pm 5\%$ S.E., $n = 4$, $p < 0.05$) than that of wild type APSCs (Fig. 4, *B* and *C*). S1P-, thrombin-, and ET-1-induced contractions were reduced by about 70% in KO versus WT reconstituted muscle fibers (Fig. 4*C*). The force generated by wild type APSCs was comparable with that of rat aortic SMCs incorporated into collagen gels further substantiating that the APSCs have a SM functional phenotype (data not shown). Furthermore, in both wild type and *Palladin* knock-out APSCs, the contraction induced by vasoactive agonists was relaxed with the ROCK inhibitor,

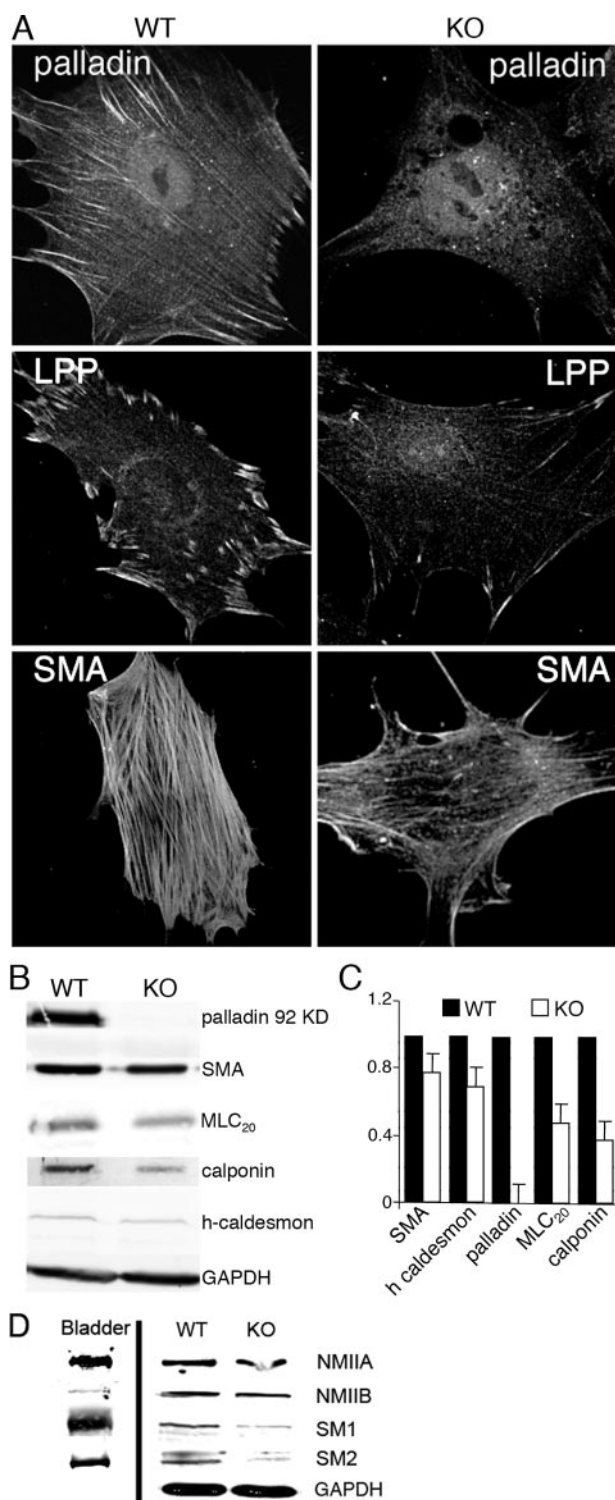


FIGURE 3. *A*, immunofluorescence results in APSCs showing reduced expression of SMA and the focal adhesion protein LPP. Palladin is localized to stress fibers in WT. The diffuse background staining in the *Palladin* KO is nonspecific. *B*, Western blotting of APSCs showing decreased MLC₂₀, calponin, and h-caldesmon protein expression in *Palladin* KO versus WT cells. Subconfluent APSCs were lysed and equal amount of protein were loaded. *C*, results of quantitative analysis of SM contractile proteins. The expression of each protein is first normalized to GAPDH and then the values for KO cells normalized to that of control cells. *D*, Western blotting showing decreased SMMHC SM1 and SM2 isoforms and no significant change of non-muscle myosin NMIIA and NMIIB in WT and KO APSCs. Mouse bladder was used as a control for MHC.

Y27632 indicating that the Ca²⁺ sensitization pathway is conserved (supplemental Fig. S2).

Agonist stimulation of SM typically induces MLC₂₀ phosphorylation, which increases actin-activated myosin ATPase activity and contraction (27, 28). Therefore, we tested whether there was any change in the MLC₂₀ phosphorylation in wild type and *Palladin* knock-out APSCs. As shown in Fig. 4C, the total MLC₂₀ in wild type was 3-fold higher than that in *Palladin* knock-out APSCs for identical protein loads indicating that the absence of *Palladin* leads to a decrease in MLC₂₀ expression in the APSCs as was seen in analyses of EBs (Fig. 2A). Lysophosphatidic acid stimulation significantly increased regulatory light chain 20 phosphorylation to the same extent in both wild type and *Palladin* knock-out APSCs. Thus the MLC₂₀ in the *Palladin* null APSCs behaved normally in terms of phosphorylation and force development but total myosin expression was reduced.

*SMC Derived from Palladin Knock-out ESC Had Normal Expression of GPCRs, ROCK, GTP-RhoA, SRF, CAS, Focal Adhesion Kinase, and Vinculin—Ca²⁺ sensitization of smooth muscle, a highly relevant physiological process, reflects an increase in MLC₂₀ phosphorylation and force independent of changes in [Ca²⁺] through inhibition of myosin light chain phosphatase or Ca²⁺ independent activation of myosin light chain kinase (28). Agonists induce Ca²⁺ sensitization through activation of G protein-coupled receptors (GPCRs), RhoA, and its downstream effectors. Therefore, we determined whether a change in the expression of Gα_{q/11}, Gα₁₂, or Gα₁₃, thromboxane receptor the active form of RhoA or Rho kinase contributed to the low force generation observed in the *Palladin* knock-out APSC. As shown in Fig. 5A, there was no detectable difference in either the expression level of these GPCRs, ROCK1 and ROCK2 or GTP-RhoA (supplemental Fig. S3). There was also no detectable difference in expression of additional focal adhesion proteins including CAS, focal adhesion kinase, vinculin or of the transcription factor, SRF (Fig. 5A). Thus, the reduced force generation in *Palladin* knock-out APSC did not appear to be due to reduced expression of these signaling and/or focal adhesion molecules.*

*Loss of Palladin Was Associated with an Increased G:F-Actin Ratio—Palladin localizes periodically along stress fibers, at focal adhesions and dense bodies, and functions as an actin cross-linking protein as well as a cytoskeletal scaffolding molecule (9). We have shown that actin dynamics regulate the expression of Palladin (4). Furthermore Miralles *et al.* (29) have shown that the SRF cofactor, MRTF-A a member of the myocardin-related transcription factors is regulated by actin dynamics and undergoes translocation from the cytosol to the nucleus in response to Rho kinase activation where it regulates SMC specific promoter activity. We did not detect a difference in nuclear and cytoplasmic MRTF-A immunofluorescence in WT and *Palladin* null APSCs. Also, MRTF-A/B mRNA expression (data not shown) and protein level (Fig. 5B) did not differ. In the *palladin* knock-out APSCs, phalloidin-stained stress fibers were less than in wild type APSCs, whereas G-actin stained with Alexa 488 DNase I was increased (Fig. 6, A and B). In contrast, there was more F-actin in wild type cells based on immunofluorescence and fractionation followed by Western*

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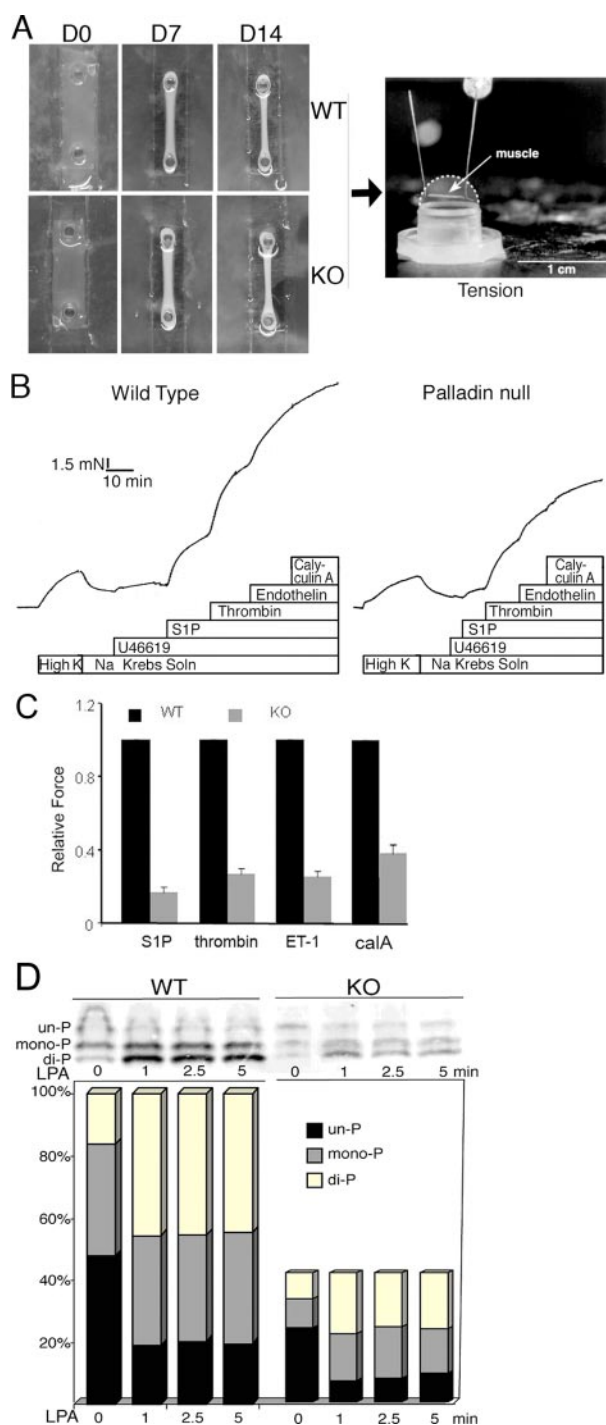


FIGURE 4. *A*, APSCs incorporated into collagen gel and imaged after 0, 7, and 14 days in culture. The *right panel* shows a strip from the collagen gel mounted for force measurements. *B*, typical isometric force measurements of wild type (WT) and *Palladin* knock-out APSCs (KO) embedded within collagen gels. Reconstituted APSC fibers were cultured in collagen three-dimensional matrix for 2 weeks. Contractions were induced with high potassium solution, 0.1 μ M of the thromboxane analogue U46619, 1 μ M S1P, 1 unit/ml of thrombin, 1 μ M endothelin-1, and 100 nM of the phosphatase inhibitor calyculin A to obtain maximal force. Force traces are representative of four similar experiments. *C*, quantitation of force measurements. *D*, lysophosphatidic acid (LPA)-induced MLC phosphorylation in APSCs. Wild type and *Palladin* knock-out APSCs were serum starved overnight, and stimulated with 1 μ M lysophosphatidic acid for 0, 1, 2.5, and 5 min. Equal amounts of cell lysates were loaded onto PAGE gels without SDS to separate the non-phosphorylated from the mono- and di-phosphorylated forms of MLC20 and transferred to polyvinylidene difluoride membranes. MLC20 was detected with anti-MLC polyclonal antibody. *D*, the *bar graph* shows quantitation of MLC20.

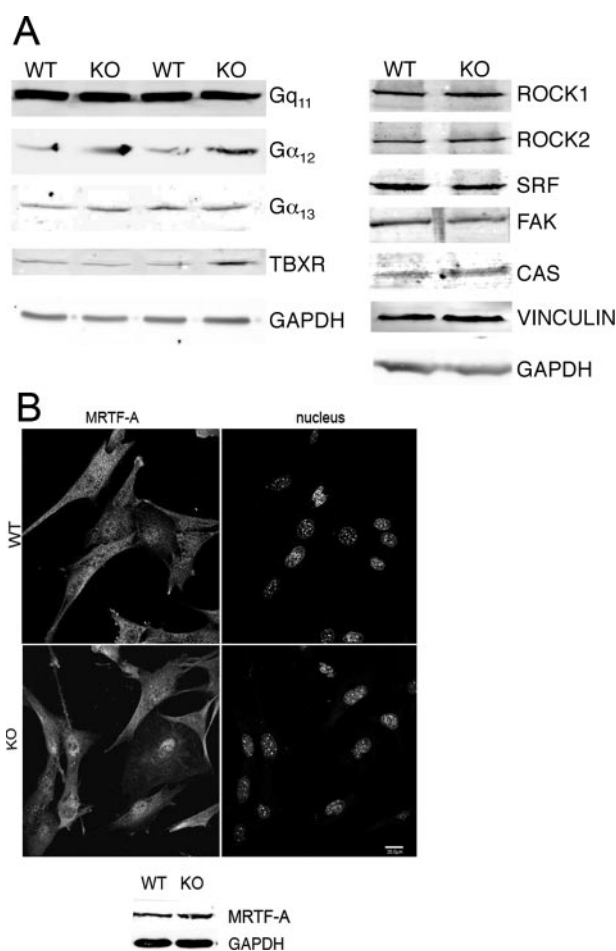


FIGURE 5. *A*, the expression of GPCRs and signaling proteins in APSCs. Equal numbers of wild type (WT) and *Palladin* knock-out APSCs were loaded on SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The proteins were detected with corresponding antibodies. No significant changes were observed in expression of these proteins. *B*, immunolabeling showing no significant difference in nuclear or cytosolic MRTF-A in *Palladin* knock-out APSCs. Scale bar, 20 μ m. The *lower panel* shows no difference in MRTF-A content by Western blot in APSCs versus WT cells normalized to GAPDH.

blotting (Fig. 6B). We propose that the lack of *Palladin* is associated with disruption of the actin cytoskeleton assembly as well as actin attachment to plasmalemmal dense bodies, and that this contributes to the reduced contractile properties of *Palladin* null APSCs. Electron microscopic examination showed a dramatic difference in cell morphology with the wild type APSCs in the collagen gels exhibiting an elongated morphology oriented longitudinally to the collagen fiber, whereas the *Palladin* knock-out cells were rounder (Fig. 6C). Due to the prevalence of ribosomes in both preparations it was difficult to evaluate the cytoskeleton. Bundles of actin filaments attached to dense bodies were observed in some wild type cells, but not in *Palladin* null fibers. Some of the wild type cells exhibited a basement membrane and caveolae, typical for SMCs. Thus, the morphology was suggestive but not definitive in showing disruption of the actin cytoskeleton in the *Palladin* null fibers. However, the increase in the G:F-actin ratio in *Palladin* null cells may regulate the expression of the SMC contractile proteins through G-actin sequestration of MRTFs.

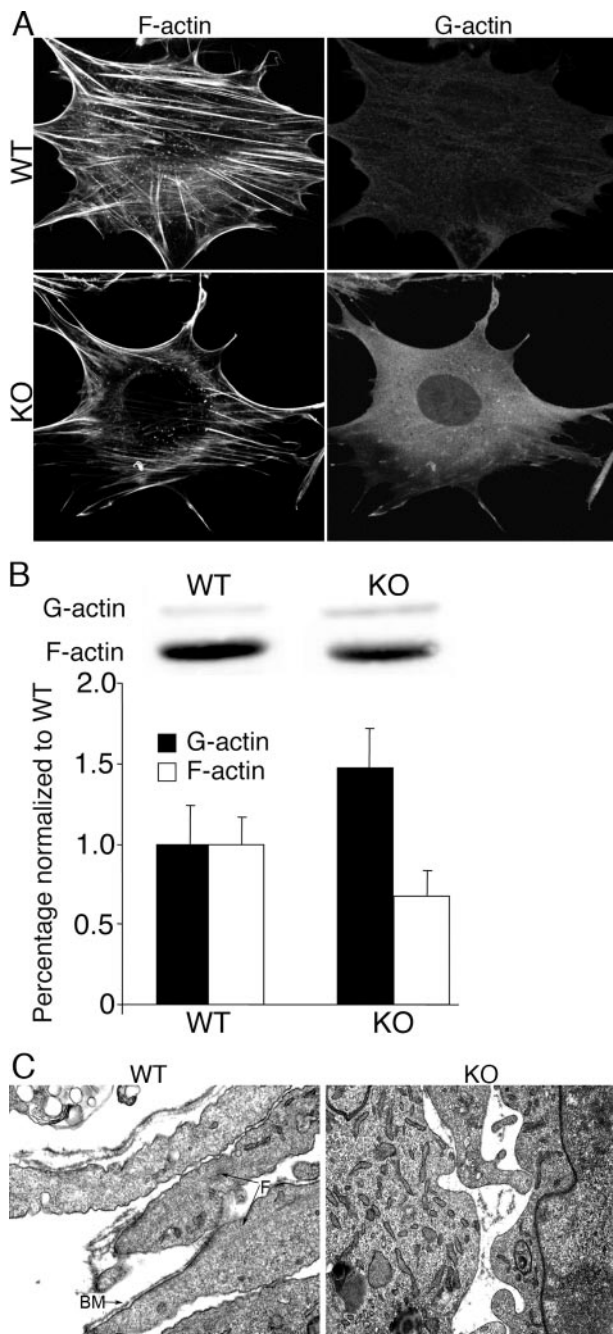


FIGURE 6. *A*, immunolabeling showing decreased F-actin and increased G-actin in *Palladin* knock-out APSC. F-actin was identified by phalloidin staining, and G-actin was stained with Alexa Fluor 488-conjugated DNase I in wild type and *Palladin* knock-out APSCs. *B*, Western blot analyses showing G:F-actin ratios. The bar graph shows the increase in the G:F-actin ratio in the *Palladin* null APSCs. *C*, electron micrographs showing the ultrastructure of APSCs incorporated into collagen gels after 14 days in culture. The WT cells were elongated and in some cases had a basement membrane, whereas the *Palladin* null cells had a more rounded morphology. *BM*, basement membrane. *F*, filament bundles.

Palladin-deficient ESC-derived SMC Showed Increased Adhesion and Decreased Migration—Actin dynamics are integral to cell-cell adhesion and cell migration is reciprocally related to adhesive forces, with strong adhesion preventing or delaying migration (30). Therefore, we studied the adhesion capability of *Palladin* knock-out APSCs on different extracellular matrices including collagen type I, fibronectin, and gelatin.

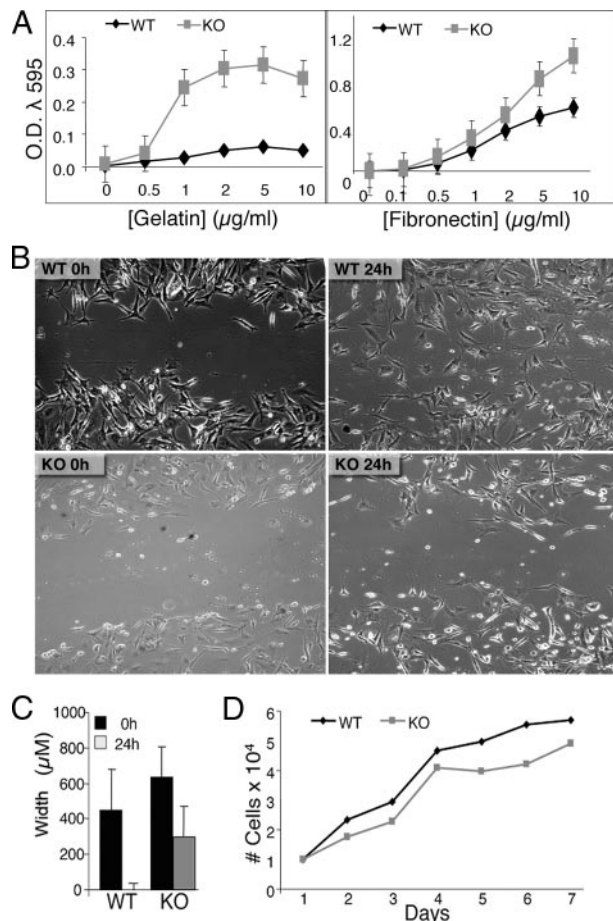


FIGURE 7. *A*, *Palladin* knock-out APSCs show increased adhesion on substrates. APSCs were cultured on purified matrix fibronectin and gelatin for 30 min. *B*, phase-contrast micrographs show the migration of APSCs in a wound assay. Images were taken at 0 and 24 h after scratching. *C*, quantification of migration data. Width = diameter of the scratch. *D*, proliferation of WT and *Palladin* null APSCs. Cells were cultured on 24-well plates. Cells were trypsinized and counted each day over 7 days. There is no difference in cell numbers in WT and *Palladin* knock-out cells.

Both wild type and *Palladin* knock-out APSCs did not attach well to collagen type I, 1 h after cell seeding. However, *Palladin* knock-out APSCs exhibited a significant increase in the number of adhering cells compared with wild type cells at 30 min post-plating on gelatin-coated plates (Fig. 7A). *Palladin* null cells also exhibited increased adherence to fibronectin-coated plates but only at concentrations of 5 $\mu\text{g/ml}$ or greater.

Palladin knock-out APSCs exhibited impaired healing of scratch wounds as compared with wild type APSC. Wild type APSCs wounds were closed at 24 h, whereas *Palladin* knock-out APSCs showed a residual gap of 300 μm ($p < 0.05$, Fig. 7B). Both cell proliferation and migration contribute to wound closure. No difference was observed in the shape of the *Palladin* null cells compared with WT. As shown in Fig. 7D, there was no detectable difference in cell proliferation between wild type and *Palladin* null APSC. However, the APSCs are selected for expression of SMA. Thus, *Palladin* null dying cells that did not express SMA would not be detected. Taken together, the preceding results confirm and extend previous work showing that *Palladin* plays an important role in regulating cell adhesion and motility (4).

DISCUSSION

Vascular SMCs are not terminally differentiated and have the ability to undergo phenotypic switching in association with vascular injury, or pathological conditions. Phenotypic plasticity of SMCs is critical for the establishment of a mature vessel, which can function to regulate vascular tone and blood vessel diameter, peripheral resistance, and the distribution of blood flow throughout the developing organism. Considerable evidence suggests that an impaired SMC phenotype during development results in defects in vascular remodeling of great arteries and congenital cardiovascular anomalies (31) but a full understanding of the complex processes underlying SMC development is far from complete. As Palladin is highly expressed in SM (4), is a key regulator of actin organization, and is required for the maintenance of normal stress fibers, focal adhesions, and migration in cultured cells (1–3), we used *Palladin* null ESCs in an EB model to investigate the role of palladin in the development of the SMC phenotype. *Palladin* null mice die at E15.5 (12) precluding their use. We found that whereas *Palladin* is not necessary for differentiation of ESCs into SMCs *per se*, as SM-MHC is expressed albeit at markedly reduced levels. We also found that the absence of *Palladin* is associated with major reductions in expression of contractile proteins, and force developing capabilities, as well as disruption of G:F-actin ratios, reduced cell migration, and increased cell adhesion.

Although the gene knock-out mouse is a powerful technology to study vascular development, animals that die *in utero* require the generation of conditional knock-out animals, which are costly. Moreover, at present, there is not a system available for selectively knocking out genes at high efficiency exclusively in SMCs (32). We used as an alternative, an *in vitro* cell differentiation model that uses multipotent ES-cell-derived EBs, which have regions of visibly contracting SMCs. Although overall morphogenesis is disrupted, EBs can recapitulate many developmental processes and form all known cell types by modifying their growth conditions (25, 26). As ESCs are capable of indefinite undifferentiated proliferation *in vitro* they also provide an unlimited supply of cells (14). Therefore, this rapid, easy to handle robust system is powerful for investigating the functional roles of candidate genes implicated in SMC development or function independent of the complicated development of the cardiovascular system. Furthermore, previous work showed that purified ESC-derived SMC populations from the EBs exhibit contractile properties equivalent to SM tissues *in vivo* (14).

The decrease in SMA, MLC20, calponin, h- and l-caldesmon protein as well as mRNA for *SM22*, *SMA*, *SM-MHC*, and *Lpp* in the EBs could be due to a reduction in differentiation of ESCs into SMCs. However, SMCs purified from *Palladin* null ESC (or APSCs) also had reduced myosin, calponin, h-caldesmon, and SMA. We cannot discriminate between the loss of these proteins being due to a defect in SMC differentiation *per se* or secondary to the loss of stress fibers. The loss in myosin is most convincingly seen in Figs. 3D and 4D, which involved evaluation of equal numbers of KO and wild type cells. These results are consistent with previously published data showing that stress fibers became faint and disordered in *Palladin* knock-out

mouse embryonic fibroblast cells (5, 12). Of note, however, APSCs were selected through use of a puromycin resistance gene under control of the promoter-enhancer region of the *SMA* gene. As such, there could have been selective loss of *Palladin* null APSCs due to reduced expression of SMA. In addition, this selection protocol may explain the paradoxical observation that *Palladin* null APSCs exhibited reduced expression of all SM marker genes examined, other than *SMA*. Indeed, this phenomenon likely resulted in our underestimating the contractile protein deficit and reduced force developing capacity of *Palladin* null APSC.

SM-MHC exists as four different isoforms produced by a single gene: SM-MHC-A, SM-MHC-B, SM1, and SM2. SM also expresses non-muscle myosins, NMIIA and NMIIIB. A SM-MHC null mouse dies shortly after birth but bladder muscle will contract in response to high potassium (33). These muscles lack the typical initial large phasic high potassium contraction but do exhibit a slow tonic contraction, about 10% of the magnitude of the WT bladder and a 2.6-fold slower shortening velocity (34). These contractions are attributed to NMIIA/B. In the *Palladin* null APSCs, there is ~50% less SM-MHC expressed compared with WT, but there is no detectable difference in NMIIA/B expression. The rates of force development were similar in the *Palladin* null and WT APSCs. Therefore, the loss of SM myosin makes the major contribution to the reduced force development in the *Palladin* null APSC reconstituted muscle fibers. The decreased calponin is not likely to contribute to the reduced force in the *Palladin* null fibers as no difference was found in maximal force in SM from calponin knock-out mice (20).

A critical question is how does Palladin regulate expression of the SMC contractile proteins? The SRF cofactors, myocardin and the myocardin family members (MRTFs) have been shown to strongly up-regulate a number of SM marker genes (35–37). Thus, one possibility is that the higher ratio of G:F-actin in the *Palladin* null knock-out SMCs results in sequestration of the SRF cofactor, MRTF-A. MRTF-A is a member of the myocardin-related transcription factors, which has been shown by Miralles *et al.* (28) to bind selectively to G-actin through its N terminus. Following Rho kinase-dependent reduction in the G:F-actin ratio, there is less binding of G-actin to MRTF-A resulting in translocation to the nucleus where it regulates SMC specific promoter activity (29). Thus the increase in G-actin in *Palladin* null APSCs may result in reduced MRTF nuclear translocation and SMC promoter activation. However, we did not detect a decrease in nuclear or an increase in cytosolic MRTF-A. Although nuclear translocations are dramatic with expressed green fluorescent protein or FLAG-tagged MRTFs (36–39), immunolabeling may not be sufficiently sensitive to detect translocation of endogenous MRTF. It is also possible that sequestration of MRTFs by G-actin may not be the sole mechanism regulating MRTF/SRF-mediated transcription (40). A recent model suggests the importance of nuclear G-actin in regulating MRTF transport and activity (41, 42). Therefore, measurements of nuclear MRTF may be insufficient for evaluation of MRTF activity. In view of these considerations and because Palladin expression strongly favors stress fiber formation and *Palladin* null cells have a significant increase in

G:F-actin, we favor the G-actin/MRTF model that leads to a decrease in nuclear MRTF activity in the absence of *Palladin*. We found no evidence for a change in GTP-RhoA activity, present in both WT and *Palladin* null cells (supplemental Fig. S3), in Rho kinase expression or in the RhoA target, SRF (Western blotting) contributing to reduced SMC marker gene expression in *Palladin* null versus WT cells. This is consistent with Palladin being downstream of RhoA and not surprisingly, that Palladin does not impact SRF expression.

The contractile state of smooth muscle reflects the ratio of activities of myosin light chain kinase and myosin light chain phosphatase, which determines the extent of MLC20 phosphorylation and actin-activated myosin II activity and can be changed by modulating the activities of the calcium-calmodulin-dependent myosin light chain kinase or myosin light chain phosphatase (28). Agonists through GPCRs, which activate the small G protein RhoA and its effector, Rho kinase, result in inhibitory phosphorylation of the regulatory subunit of myosin light chain phosphatase leading to an increase in MLC20 phosphorylation and force, independent of a change in $[Ca^{2+}]_i$, a process termed Ca^{2+} sensitization (28). In *Palladin* knock-out SMC-like cells, force generation in response to vasoactive agonists is significantly lower compared with that of wild type cells. This ~60% decrease likely reflects the decrease in total myosin cross-bridges and the number of active phosphorylated heads in the knock-out cells. The extent of MLC20 phosphorylation was similar in the KO and wild type. Thus, the cells did not compensate by increasing myosin light chain kinase or decreasing myosin light chain phosphatase activity. In addition, the lower total myosin and the higher ratio of G:F-actin in knock-out SMCs would result in fewer actin sites and myosin cross-bridges for force development. Ca^{2+} sensitization signaling pathways could also be altered yet GPCRs and ROCKs are normally expressed in the *Palladin* knock-out SMCs (Fig. 5), and active GTP-RhoA is the same as that of wild type SMCs (supplemental Fig. S3) making alterations in Ca^{2+} sensitization signaling pathways less likely to contribute to the reduced force.

We show here that the *Palladin* knock-out APSCs have less stress fibers and a decreased motility, consistent with published results (4, 43), further affirming a role of Palladin in invasive motility such as in tissue repair, embryonic development, and cancer invasion. We observed in *Palladin* null APSCs increased adhesion on fibronectin and gelatin contrary to others (12). The difference may be because they used embryo fibroblasts isolated from E12.5 embryos, a later stage in development than the early stage ESC/EB-derived SMC like cells, used in the present study. The delayed migration of the *Palladin* null APSCs also supports the theory that cell migration is tightly related to the adhesive forces, and strong adhesion can prevent or delay migration (24).

In summary, we show that Palladin is required for expression of the full complement of myosin and actin necessary for maximal contractile responses and that alterations in Palladin expression change the G:F-actin ratio, which in turn may regulate the myocardin-related cofactors and transcription of the contractile proteins. We further demonstrate that ESC/EB models are amenable to study of SM development and contractile properties. The molecular mechanism(s) whereby pal-

ladin regulates the expression of contractile proteins and whether Palladin contributes significantly to the contractile properties and to development of the vasculature *in vivo* under normal and pathological states will be the direction of our future investigations.

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